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Effects of Inoculating Earthworms on Degradation of Pyrene in the Rhizosphere Soils Growing *Sorghum sudanese*

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The potentials of Sudan grass, with or without inoculating earthworms, on the degradation of hydrophobic organic contaminants (HOCs) in soils were estimated by pot experiments. Results showed that plantation of vegetation enhanced loss of HOCs at initial contents ranging from 20.24 to 321.42 mg/kg. During the 70-d incubation, about 801.84~539.99‰ of pyrene was removed from planted soils, and only 242.28~122.79‰ degradation of pyrene occurred in non-planted soils. After inoculating earthworms, the dissipation ratios of pyrene in planted soils were increased up to 863.94~609.63‰, which was 77.27~129.14‰ higher than those in corresponding soils without earthworms. Among all possible pathways, contribution of plant-microbial interactions on dissipation of pyrene was the most significant, either with (456.73‰) or without inoculating earthworms (515.58‰) and were the primary means of contaminant degradation. Results suggested a feasible way for the establishment of high efficiency rhizoremediation of HOCs with inoculating earthworms, which may be especially beneficial for reinforcing removal of HOCs containing more benzene rings in molecules.

Key words: Rhizoremediation, Pyrene, Plant-microbial interactions, Sudan grass, Earthworm.

Phytoremediation is a promising approach to soil remediation due to its convenience, cost-effectiveness and environmental acceptability. Plants may contribute to the dissipation of organic contaminants through an increase in the number of microbes, improvement of soil properties and structure, promotion of humification and adsorption of pollutants in the rhizosphere, but the impact of each process has not been clearly elucidated, and remediation efficacy varies greatly among plant species depending on the differences of soil conditions and the physicochemical nature of contaminants [1]. However, there also exist many limitations for large-scale application of this technology. One serious limitation is that many plant species are sensitive to hydrophobic organic contaminants (HOCs) including polycyclic aromatic hydrocarbons (PAHs), their growth could be retarded by these pollutants; thus the biomass accumulated are not enough to extent that is meaningful to soil remediation; simultaneously, in most contaminated soils, microorganisms are also depressed so that there are not enough bacteria to facilitate HOCs degradation or to support plant growth [2]. In addition, HOCs in soils usually exhibit low bioavailability to both microorganisms and plants due to their strong affinity to the soil matrix, especially to soil organic matter [3], which would limit the application of phytoremediation.

Earthworms are common representatives of the soil macrofauna, which live in close contact

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with HOCs. It is well established that earthworms are beneficial to the improvements of physicochemical properties and structure of soil, to the promotion of vailability of mineral nutrients to plants [4], and to the increase of microbial populations and biologically active metabolites such as plant growth regulators. We hypothesized that earthworms casting and burrowing activity may also enhance effects of rhizoremediation by improving oxygen diffusion conditions. In fact, oxygen availability is still the rate-limiting factor for effective rhizoremediation because HOCs degradation by certain soil bacteria, including the mycobacteria, requires transformation through dioxygenases. We also expected an increased HOCs degradation and plant establishment on contaminated sites as well as increased resistance to potentially phytotoxic effects of the pollutants if earthworms introduced. Although the potential role of earthworms in the breakdown of a wide range of organic residues have been well demonstrated[5], few studies have been carried out concerning the impact of earthworm activity on rhizoremediation efficiencies of HOCs in soils.

In this study, effect of Sudan grass (*Sorghum sudanense*), with or without earthworms, on the dissipation of pyrene (Pyr) in soils was investigated and each removal pathway of Pyr in the process of remediation was compared in order to understand enhancement potentials of earthworm on phytoremediation by Sudan grass, thus to establish an efficient way for the remediation of HOCs in soils.

MATERIALS AND METHODS

Chemicals

Pyrene (Pyr), as representative of HOCs, was obtained from Sichuan University with a purity > 98.5%, molecular weights of 202.26 g/mol and 4.88 of logK_{ow} (K_{ow}, octanol-water partition coefficient).

Contaminated soils

Soils tested were collected from 6~18 cm horizontal with pH 6.78, 1.46% organic matters and originally free of PAHs. After being air-dried and sieved through a 2 mm mesh, soils were spiked with Pyr in acetone. When acetone evaporated off, spiked soils were progressively mixed with unpolluted soils and homogenized. The initial Pyr in soils were measured and followed as: C_0 (free PAHs), C_1 (20.24 ± 0.94 mg/kg), C_2 (39.58 ± 1.51 mg/kg), C_3 (79.86 ± 2.37 mg/kg), C_4 (160.64 ± 3.05 mg/kg) and C_5 (321.42 ± 4.93 mg/kg). Treated soils were packed into pots (2.0 kg) and equilibrated 4d under 45% of the water holding capacity (WHC).

Plant

After surface was sterilized in 10% H_2O_2 for 10 min and rinsed with sterile distilled water, Sudan grass seeds were germinated and grown in moist perlite, and seedlings of uniform size were transplanted to the designated greenhouse pots 7d after emergence.

Earthworm cultures

Healthy earthworms (*Pheretima sp.*) individuals, which were selected from a population of similar ages, were separated into 10 sub-cultivation units containing 25 kg of clean soil with a density of 10 earthworms per kilogram. Under controlled conditions (20±2°C), earthworms were fed with separated cattle solids, which were dried to drive off ammonia and kill unwanted invertebrates, crumbled and thoroughly mixed into the cultures at a rate of 75 g/kg for 30d. Juveniles of analogous size were selected for the experiment. Only individuals without fully developed clitellum were used in the study in order to ensure a uniform set of organisms tested.

Experimental design

Experiments were conducted in an intelligent greenhouse. They were divided into 2 groups according to studied objects, namely no earthworms group (Group A) and inoculating earthworms group (Group B). In Group A, four different treatments with five replicates were performed :a) treatments 1(CK_1), unplanted microbe-inhibited soils, where 0.1%NaN_3 was added to inhibit the microbial activity[6], b)treatments 2(CK_2), unplanted soils, c)treatments 3(TR_3), planted microbe-inhibited soils added 0.1%NaN_3, and d)treatments 4(TR_4), planted soils. Seedlings were thinned 5d after transplanting to eight plants per pot. WHC in soils was checked and adjusted regularly with sterilized water to 50% [6].

In Group B, the same established treatments and culture conditions were used. After washing with distilled water, six earthworms (2.4~2.6 g wet mass, 7~8 cm in length) were introduced into soils at the beginning of experiments. Though no food was added during the 70-d experiment, earthworms increased up to 21% in mass averagely, indicating that conditions were suitable and organic matter in soils was a viable food source.

Soils and plants were destructively sampled after 70d after transplanting. Shoot and root tissues, separated from soil, were washed with distilled water and dried with filter paper. The plant materials and soil samples were stored at –40.0°C to prevent microbial degradation of contaminants. An aliquot of each soil or plant sample was weighed, dried at 105.0°C for 24h, and weighed again.

Determination of HOCs.

The Pyr content in the extractant was determined using the high performance liquid chromatography method with ultraviolet detection after a preliminary sample treatment with ultrasonic techniques. The detailed methods to extract Pyr have been described [6], and these from earthworm tissue followed Johnson[7].

Prior to use, all methods were tested for efficiency of recovery. For PAHs-amended soils, plant and earthworm tissue samples, recovery averaged 93.52 % (n=9, RSD<6.37%; RSD, relative standard deviation), 93.23% (n=9, RSD< 5.49%) and 89.24% (n=9, RSD< 6.71%) respectively.

Data analyses

The obtained data were analyzed using SPSS version 12.0, and levels of significance were assessed with Duncan's multiple-range test (DMRT, $p \le 0.05$).

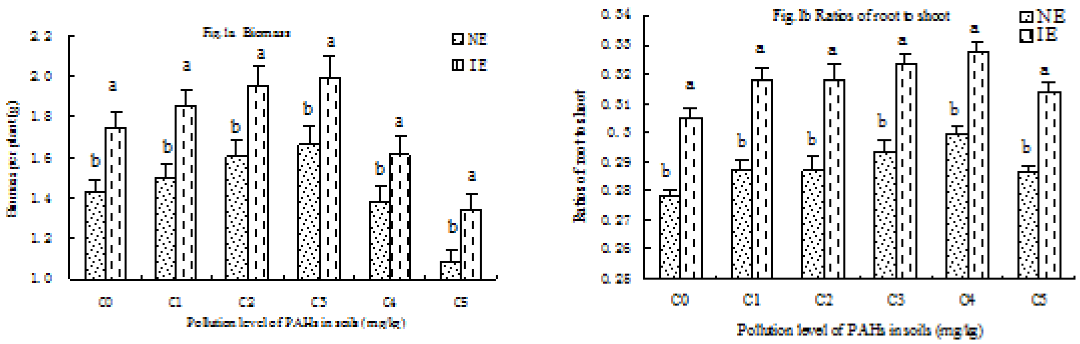
For every treated pot, the dissipation rate (D) of Pyr in soils was calculated as $D = (C_0 - C_t) \times 1000‰/ C_0$, where C_0 was the initial contents of Pyr in soils, and C_t denoted residual Pyr.

For given biotic & abiotic factor i, its contribution rate (T_i) to the dissipation of PAHs in the process of phytoremediation was expressed as $T_i = D_i \times 1000‰/ W \cdot C_0$, where D_i was the removed amount of PAHs by given factor i, and W denoted the weight of spiked soils in pot. Obviously, the D value should be theoretically equal to the sum of contribution rates of all factors.

RESULTS

Plant biomass

As shown in Fig.1, Sudan grass showed no signs of stress and produced abundant biomass in spiked soils. Throughout the experiment, the total mass of Sudan grass in soils with low (C_1), medium (C_3) and high (C_5) initial contents of Pyr were almost equal to those of unspiked soils (1.43g) with the almost same ratios of root to shoot biomass (0.284). Though biomass of plants grown in high pollution level soils (C_5) was slightly decreased, all plants tested did not also show any visible sign of toxicity, indicating that establishment of vegetation in these soils is feasible. Although the weights of roots or shoots of Sudan grass grown in soils inoculated earthworms (IE) were on average 27.2% and 16.6% greater than those grown in spiked soils, differentiation in the total biomass of seedlings growing in variously spiked soils (C_0~C_5) between with and without inoculating earthworms (NE) was insignificant (n=30, P>0.05). However, their ratios of root to shoot biomass tended to be significantly greater in the presence of earthworms (n=30, P<0.05), indicating that earthworm activity was more favorable to the growth of the root systems.



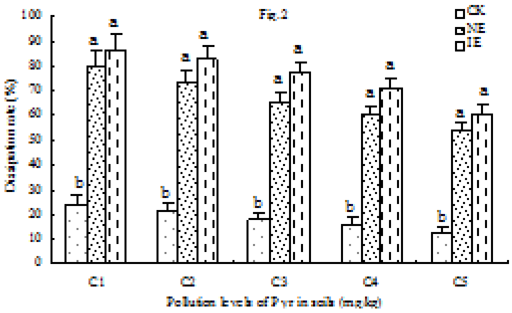
Note: IE and NE denoted the Pyr-spiked soils with and without inoculating earthworms, respectively

Fig. 1. Biomass and ratios of root to shoot of plants grown in treated soils

Dissipation of HOCs

On the whole, residual Pyr in soils inoculated with earthworms were always lower than those without earthworms for the same treatment. Within the same group, removal of Pyr among different treatments showed a consistent descending order: $CK_1 \leq TR_3 < CK_2 < TR_4$. With an increase of initial contents of Pyr in soils, their *D* value linearly declined for the same treatment. As shown in Fig.2, the presence of plants apparently enhanced the removal of Pyr in soils with or without inoculating earthworms. During the experiment, approximately 757.33‰ (in a range of 863.94~609.63‰) of Pyr was removed from the TR_4 with earthworms as compared with 665.19‰ (801.84~539.99‰) degradation without earthworms, while only 182.38‰ (242.28~122.79‰) removal occurred in CK_2 without earthworm at concentrations of C_1~C_5. Of the spiked soils, the Pyr dissipation rates in TR_4 with earthworms were the highest, indicating that introducing earthworms into phytoremediation system could enhance removal of Pyr to a certain extent.

Though the dissipation of Pyr in all tested soils with earthworms were larger than those without earthworm, the extent of enhanced removal varies greatly among five pollution levels. As shown in Fig.3, the enhanced dissipation of Pyr in soils with medium pollution level (C_3) was the highest, which were up to 120.11‰ in the vegetated soils (TR_4). Comparatively, fewer extents of removal enhanced occurred in soils with low (C_1) or high (C_5) pollution level, only 62.01‰ and 69.60‰ additional removal, respectively. On a whole, extents of enhanced dissipation of Pyr were up to 92.06‰ during 70-d rhizoremediation.



Note: CK, IE and NE represent unplanted spiked soils, and planted soils with and without inoculating earthworms, respectively

Fig.2. Dissipation rates of pyrene in soils under different treatments

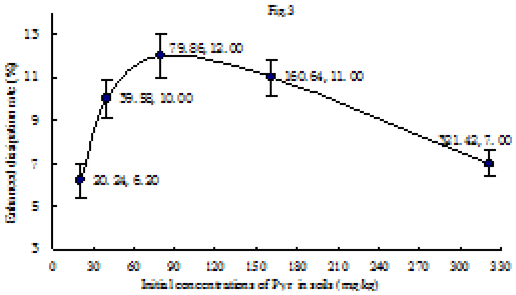


Fig.3. Enhanced dissipation rates of pyrene in soils at different pollution levels by earthworm

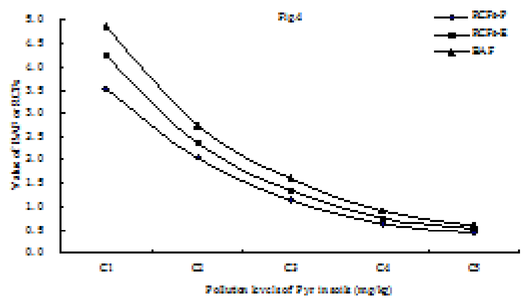
HOCs accumulated in plant and earthworm tissues

As expected, accumulation levels of Pyr in plant and earthworm tissues have a positive correlation with their pollution levels; Pyr contents in plant tissues are always lower in soils inoculated earthworms than those without earthworms. With the increase of pollution levels in soil, Pyr contents in roots and shoots of plants growing in soils without earthworms increased from 14.17 to 72.26 mg/kg and from 2.08 to 13.79 mg/kg while increased from 9.69 to 58.59 mg/kg and from 1.32 to 10.36 mg/kg when inoculating earthworms, respectively.

Based on residual HOCs in soils and their accumulation levels in roots of plant and earthworm tissues by the end of the experiment, RCFs (root concentration factors, defined as the ratio of HOCs concentration in roots and one in soils) and BAF (bioaccumulation factor, equaled to HOCs concentration in earthworm tissues divided by those in soils) of Pyr in soils were calculated as shown in Fig.4. RCFs of Pyr in soils without earthworms were 0.49~3.54, slightly lower than those inoculated earthworms (0.55~4.25). Generally, BCFs of Pyr tended to decrease with the increase of contaminants concentration in soils. Results are similar to BAF by earthworm tissues, which varied in a range of 0.56~4.88.

Removal pathways of HOCs

Removal of HOCs in the plant-soil system



Note: RCFs-E and RCFs-P denoted root concentration factors in soils with and without inoculating earthworms, respectively

Fig. 4. RCFs or BAF of pyrene as a function of PAHs concentrations in soils

attributed to both biotic pathways, e.g., plant accumulation, plant metabolism, microbial degradation and plant-microbial interactions, and abiotic pathways, e.g., leaching, volatilization, photodegradation and irreversible sorption, etc. If the variation of abiotic loss of HOCs between planted and unplanted soils was negligible, HOCs removed in CK_2 should be equal to the sum of abiotic loss and microbial degradation while loss of HOCs in CK_1 attributed to abiotic loss. Thus, removal of Pyr in soils could approximately be expressed as

$$D_1 = T_a$$
$$D_2 = T_a + T_m$$
$$D_3 = T_a + T_c + T_d$$
$$D_4 = T_a + T_c + T_d + T_m + T_{pm}$$

Where D_1, D_2, D_3 and D_4 were the removed amount of Pyr in CK_1, CK_2, TR_3 and TR_4, and T_a, T_m, T_c, T_d and T_{pm} represented the contribution of abiotic loss, microbial degradation, plant accumulation, plant metabolism and plant-microbial interactions on removal of Pyr, respectively.

As to soils inoculated earthworms, effects of direct accumulation and enhanced removal of Pyr from earthworms should be also taken into account. Then, enhanced D value of Pyr in CK_1, CK_2, TR_3 and TR_4, could be respectively expressed as

$$DD_1 = T_a^{\,e} + T_e$$
$$DD_2 = T_a^{\,e} + T_m^{\,e} + T_e$$
$$DD_3 = T_a^{\,e} + T_c^{\,e} + T_d^{\,e} + T_e$$
$$DD_4 = T_a^{\,e} + T_c^{\,e} + T_d^{\,e} + T_m^{\,e} + T_{pm}^{\,e} + T_e$$

Where T_e represented the contribution of earthworm accumulation on Pyr dissipation, and $T_a^{\,e}$, $T_m^{\,e}$, $T_c^{\,e}$, $T_d^{\,e}$ and $T_{pm}^{\,e}$ did the enhanced amount of T_a, T_m, T_c, T_d and T_{pm} with earthworms, respectively. Based on Pyr detected in leachate, soils, plant and earthworm tissues, contribution rate of each pathway on removal of Pyr was calculated as Table 1.

Table 1. Contributions of biotic & abiotic factors to dissipation of PAHs in soils without and with inoculating earthworms among treatments (‰)

Factors	C_1	C_2	C_3	C_4	C_5
Abiotic loss	32.07 (1.17)	29.28 (1.93)	25.64 (2.28)	22.61 (1.51)	18.16 (1.16)
Microbial degradation	210.23 (14.46)	182.62 (27.53)	152.25 (37.61)	134.34 (34.15)	104.62 (18.47)
Plant accumulation	1.06 (-0.003)	0.72 (-0.005)	0.62 (-0.007)	0.37 (-0.003)	0.29 (-0.001)
Plant metabolism	49.24 (6.80)	39.46 (5.62)	21.82 (3.86)	9.81 (3.14)	5.14 (2.18)
Earthworm accumulation	0 (1.59)	0 (0.97)	0 (0.72)	0 (0.51)	0 (0.37)
Plant-microbial interactions	509.24 (37.98)	476.51 (63.85)	452.19 (75.63)	435.83 (69.37)	411.78 (47.42)

Note: data in bracket were the enhanced T_i of given factor i when inoculating earthworms

In the entire experiments, no leachate was produced when WHC in soils was maintained at about 50%, and abiotic loss by leaching was insignificant. As seen from table 1, the D value of Pyr in microbe-inhibited pots, i.e., CK_1, was 18.16~32.07‰ (M=25.58‰), indicating that abiotic loss was a relatively minor pathway for the dissipation of Pyr. However, contribution of microbial degradation on removal of contaminants was notable, which was up to 104.62~210.23‰ (M=158.76‰). In this case, role of plant accumulation in dissipation of Pyr was inappreciable compared to the total loss of HOCs. During the experiment, the amount of Pyr accumulated by Sudan grass only accounted for 0.29~1.06‰ (M=0.61‰) from C_1 to C_5 treatment, which were less than 0.92‰ of Pyr degraded averagely while one derived from plant metabolism was in the range of 5.14~49.24‰ (M=25.09‰), which were less than 38.22‰ of Pyr degraded in soils. Among all removal pathways, the plant-microbial interactions contributed the most part for rhizoremediation of HOCs, which removed up to 456.73‰ (in a range of 411.78~509.24‰) from spiked soils averagely, illuminating that the predominant pathway responsible for the dissipation of HOCs in soils was the plant-microbial interactions.

As shown in table 1, enhanced dissipation of Pyr in soils inoculated earthworms varied greatly among removal factors. On the whole, the enhanced extent of plant-microbial interactions and microbial degradation were higher, which was up to 58.85‰ and 26.44‰ on average while comparing with the much lower abiotic loss, whose contributions enhanced only accounted for 1.61‰. It was notable that the contribution rate of plant accumulation to Pyr was slightly lower in soils inoculated earthworms than in soils without earthworms, which was consistent with the experimental facts that with inoculating earthworms, Pyr concentrations in plant tissues were always lowered at the same soil concentrations.

DISCUSSIONS

Phytoremediation has been demonstrated to be a feasible cleanup alternative for HOCs in rhizosphere soils, the selection of the plant species is critically important to the success of phytoremediation[8]. In this study, plant-mediated HOCs dissipation was investigated, and loss of Pyr in variously spiked soils (C_1~C_5) with Sudan grass was 801.84~539.99‰ of the soil with these chemicals, which were 2309.22~3397.34‰ larger than those in unplanted soils. The high dissipation ratios of HOCs in planted soils, the evident dissipation promotion of these compounds in the presence of vegetation, and the healthy growth of the plant in variously spiked soils suggest the feasibility of remediation of HOCs in soils using Sudan grass.

After inoculating earthworms, the dissipation of Pyr in variously unplanted soils (C_1~C_5) were 257.93~142.43‰, which were 64.53~159.94‰ higher than those in corresponding soils without earthworm, and in soils with Sudan grass were up to 863.94~609.63‰, which were 77.27~129.14‰ higher than those in corresponding soils without earthworm, respectively. Previous studies [9] also showed that the activity of earthworms could enhance the dissipation of HOCs by stimulating the amount and activity of soil microbial biomass, improving their acclimation and enhancing their adaptation. Furthermore, earthworms reinforced the dissipation of HOCs by aerating the soil or providing nutrients for the soil microorganisms [10]. These results also suggested that the extent of removal enhanced in soil with medium (C_3) pollution level, either planted or unplanted, appeared to be the strongest, probably because of the low bioavailability of dissipation of HOCs due to adsorption to soil particles, and lack of adequate microbial activity when pollution levels are too low or too high, which went against the dissipation of HOCs under such conditions.

It is noteworthy that the amount of Pyr accumulated by Sudan grass growing in soils inoculated earthworms were always less than those without earthworms, and Pyr concentrations in plant tissues, irrespective of root and shoot, were always slightly lower at the same soil concentrations, implying that inoculating earthworms seemed to be beneficial to decreasing HOCs accumulations in plant tissues to a certain extent, which could be especially beneficial for relieving potentially ecological risks.

CONCLUSIONS

In conclusion, inoculating earthworms apparently reinforced the dissipation of Pyr in planted soils at initial contents ranging from 20.24 to 321.42 mg/kg. During the 70-d experiment, after inoculating earthworms, loss of Pyr in variously spiked soils (C_1~C_5) with Sudan grass were up to 863.94~609.63‰, which were 92.06‰ larger than those in corresponding soils without earthworm averagely, which suggested feasibility of the establishment of inoculating earthworms for improvement of rhizoremediation of HOCs. However, it remains to be seen whether the dissipation of HOCs in the plant-soil system inoculated earthworms is as effective under large-scale field conditions as it appears under laboratory conditions.

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